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Montanide™ IMS 1313 N VG PR nanoparticle adjuvant enhances antigen-specific immune responses to profilin following mucosal vaccination against *Eimeria acervulina*☆

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ABSTRACT

This study investigated protection against *Eimeria acervulina* (*E. acervulina*) following vaccination of chickens with an *Eimeria* recombinant profilin in conjunction with different adjuvants, or by changing the route of administration of the adjuvants. Day-old broilers were immunized twice with profilin emulsified in Montanide™ IMS 1313 N VG PR adjuvant (oral, nasal, or ocular routes), Montanide™ ISA 71 VG adjuvant (subcutaneous route), or Freund's adjuvant (subcutaneous route) and orally challenged with virulent *E. acervulina* parasites. Birds orally immunized with profilin plus IMS 1313 N VG PR, or subcutaneously immunized with profilin plus ISA 71 VG, had increased body weight gains compared with animals nasally or ocularly immunized with profilin plus IMS 1313 N VG PR, or subcutaneously immunized with profilin plus Freund's adjuvant. All adjuvant formulations, except for IMS 1313 N VG PR given by the nasal or ocular routes, decreased fecal parasite excretion and/or reduced intestinal lesions, compared with non-vaccinated and infected controls. Compared with animals vaccinated with profilin plus Freund's adjuvant, chickens immunized with profilin plus IMS 1313 N VG PR or ISA 71 VG showed higher post-infection intestinal levels of profilin-reactive IgY and secretary IgA antibodies. Finally, immunization with profilin in combination with ISA 71 VG was consistently better than profilin plus IMS 1313 N VG PR or Freund's adjuvant for increasing the percentages of $CD4^+$, $CD8^+$, $BU1^+$, $TCR1^+$, and $TCR2^+$ intestinal lymphocytes. These results indicate that experimental immunization of chickens with the recombinant profilin subunit vaccine in conjunction with IMS 1313 or ISA 71 VG adjuvants increases protective mucosal immunity against *E. acervulina* infection.

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1. Introduction

Avian coccidiosis is a widespread and economically important disease caused by infection of the intestine by

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protozoan parasites from the genus *Eimeria* (Lillehoj and Lillehoj, 2000). Live vaccines containing attenuated parasite strains are commercially available to control chicken coccidiosis. Subunit vaccines, such as synthetic peptides and recombinant proteins, in general possess limited immunogenicity and remain, for the most part, in investigational development. Incorporation of novel adjuvants and/or use of alternative delivery systems have shown promise in augmenting the immunogenicity of coccidiosis subunit vaccines and controlling *Eimeria* infection under selected experimental conditions (Jang et al., 2010, 2011; Lee et al., 2010; Sharman et al., 2010). Furthermore, discovery of novel adjuvants that can potentiate mucosal immunization against avian coccidiosis will facilitate the development of an efficacious vaccination strategy feasible for poultry industry (oral, spray).

Adjuvants were originally identified in the 1920s by Gaston Ramon, a French veterinarian working at the Pasteur Institute in Paris (Ramon, 1925). Subsequently, a variety of diverse chemical compounds and formulations have been identified that stimulate the immune system's response to a target antigen without themselves conferring immunity. These include incomplete and complete Freund's adjuvants (IFA and CFA), lipid A derivatives, purified saponins, and aluminum hydroxide (Lacaille-Dubois and Wagner, 1996; Oda et al., 2004). Our prior study demonstrated that subcutaneous vaccination of chickens with an *Eimeria* recombinant profilin formulated as a water-in-oil emulsion with the Montanide™ ISA 71 VG (ISA 71 VG) adjuvant enhanced protective immunity to coccidiosis when given prior to oral infection with live parasites, compared with adjuvant-free immunization (Jang et al., 2010). In addition, the profilin/ISA 71 VG adjuvant combination induced protective immunity against multiple *Eimeria* spp. compared with vaccination with profilin alone, an effect that cannot be achieved using live, attenuated parasite vaccines without incorporation of all of the relevant coccidia species into the vaccine (Jang et al., 2011).

ISA 71 VG consists of a blend of oil plus an ester of mannitol and oleic acid with unique emulsifying properties due to its polar sugar group, non-ionic polar sugar group and specificity of the fatty acid chains (Riffault et al., 2010). While the Montanide™ ISA series of water-in-oil adjuvants has shown benefit in enhancing immunity against a variety of human and veterinary infectious pathogens (Aucouturier et al., 2001, 2002, 2006), vaccine delivery to mucosal surfaces under field conditions is generally more efficacious using aqueous solutions. Further, water-in-oil emulsions may not be compatible with vaccination at diverse mucosal epithelia as the continuous phase of these vaccines formulations is oil. Montanide™ IMS 1313 N VG PR (IMS 1313) adjuvant consists of a water-dispersed liquid nanoparticles combined with an immunostimulating compound. Because this adjuvant has an aqueous phase, it is suitable as a mucosal delivery vehicle. Montanide™ IMS 1313 is also suitable for mass vaccination that can be used in intensive poultry industry via spray, shower or drinking water (Riffault et al., 2010).

Therefore, the current investigation compared oral, nasal, and ocular immunizations of profilin in combination with the IMS 1313 adjuvant, comprising water-dispersed liquid nanoparticles combined with an immunostimulating compound, with profilin plus ISA 71 VG or profilin plus the classical Freund's adjuvant given subcutaneously.

2. Materials and methods

2.1. Experimental animals

One day-old male broiler chickens (Ross/Ross, Longenecker's Hatchery, Elizabethtown, PA) were housed in Petersime starter brooder units and provided feed and water *ad libitum*. At 14 days post-hatch, the chickens were transferred to larger hanging cages housing 2 birds per cage for the remainder of the experiment. All protocols were approved by the Beltsville Area Institutional Animal Care and Use Committee.

2.2. Recombinant profilin

The profilin gene was originally cloned by immunoscreening an *E. acervulina* cDNA library using a rabbit antiserum against *E. acervulina* merozoites (Song et al., 2000). The 1086-base pair profilin cDNA was subcloned into the pMAL plasmid with an NH_2-terminal maltose-binding protein epitope tag and a Factor Xa protease cleavage site between maltose-binding protein and profilin (Ding et al., 2004). Transformed *Escherichia coli* DH5α bacteria were grown to mid-log phase, induced with 1.0 mM of isopropyl-β-D-thiogalactopyranoside for 3 h at 37 °C, collected by centrifugation, and disrupted by sonication on ice (Misonix, Farmingdale, NY). The recombinant profilin was isolated on an amylose affinity column (New England Biolabs, Beverly, MA) according to the manufacturer's instructions, digested with Factor Xa to release profilin from the solid phase, and repassed through a second amylose column to remove contaminating maltose-binding protein. Final purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with profilin-specific rabbit antibody (Ding et al., 2004).

2.3. Experimental adjuvants and vaccine formulation

Montanide™ IMS 1313 N VG PR (SEPPIC, Puteaux, France) is a dispersion of nanoparticles in an aqueous phase containing an immunostimulating component and Montanide™ ISA 71 VG is ready to emulsify adjuvant to generate a water-in-oil emulsion (Aucouturier et al., 2001). Purified profilin was mixed with IMS 1313, CFA, or IFA at 50:50 (wt:wt, profilin:adjuvant) ratios. ISA 71 VG was mixed with profilin at a 30:70 ratio.

2.4. Parasites

The strain of *E. acervulina* used in this study was originally developed and maintained at the Animal Parasitic Diseases Laboratory of the Animal and Natural Resources Institute (Beltsville, MD) (Jang et al., 2010). Sporulated oocysts were cleaned by flotation on 2.5% sodium hypochlorite, washed 3 times with PBS, and enumerated using a hemocytometer.

Table 1
Experimental design.

Experimental group	Number of birds	Profilin (μg/bird)	Adjuvant	Delivery route[a]	Challenge
1	18	–	–	–	–
2	18	–	–	–	1.0×10^4 *E. acervulina*
3	18	50	CFA/IFA	SC	1.0×10^4 *E. acervulina*
4	18	50	ISA71VG	SC	1.0×10^4 *E. acervulina*
5	18	50	IMS1313	O	1.0×10^4 *E. acervulina*
6	18	50	IMS1313	N	1.0×10^4 *E. acervulina*
7	18	50	IMS1313	OC	1.0×10^4 *E. acervulina*

[a] SC, subcutaneous; O, oral; N, nasal; OC, ocular. Chickens were immunized twice with the indicated profilin/adjuvant combinations at 7 and 14 days post-hatch by the designated delivery routes. One week post-secondary immunization, the birds were challenged with or without *E. acervulina*.

2.5. Experimental design

The experimental design is summarized in Table 1 and Fig. 1. At 1 week of age, chickens were randomly divided into 7 groups (18 birds/group) and orally immunized (individual gavage method), nasally (individual nasal drop), or ocularly (individual eye drop) with 50 μg of recombinant profilin plus IMS 1313 (groups 5–7) or subcutaneously with 50 μg of profilin plus ISA 71 VG (group 4) or CFA/IFA (group 3). Control chickens were subcutaneously immunized with PBS (groups 1 and 2). At 7 days post-primary immunization, chickens were immunized with PBS or 50 μg of profilin plus the homologous adjuvants, except for CFA which replaced with IFA. At 7 days post-secondary immunization, groups 2–7 were orally infected with 1.0×10^4 sporulated *E. acervulina* oocysts. Non-infected, PBS-injected control birds (group 1) were used as negative controls.

2.6. Measurement of body weight gains, fecal parasite excretion, and lesion scores

Body weights were measured between 0 and 10 days post-infection. For determination of fecal parasite shedding, fecal samples were collected daily between 5 and 10 days post-infection and oocysts were individually enumerated using a McMaster counting chamber as described (Ding et al., 2004). Lesion scores were determined at 6 days post-infection on a scale between 0 (none) and 4 (high) in a blinded fashion by 3 independent observers as described (Johnson and Reid, 1970).

2.7. Measurement of intestinal IgY and IgA levels

At 3 days post-second immunization and 3 days post-infection, birds were sacrificed by cervical dislocation, the duodenum was removed, cut longitudinally, and incubated for 4 h on ice in 10 ml of ice-cold PBS containing 0.05 trypsin inhibitory units/ml of aprotinin, 5.0 mM EDTA, 2.0 mM phenylmethylsulfonyl fluoride, and 0.02% sodium azide (Sigma, St. Louis, MO). The intestinal washes were clarified by centrifugation and stored at −20 °C prior to enzyme-linked immunosorbent assay (ELISA) for profilin-reactive IgY or secretory IgA (sIgA) antibody levels as described (Lillehoj et al., 2005). Briefly, 96-well microtiter plates were coated overnight with 1.0 μg/well of purified profilin. The plates were washed with PBS containing 0.05% Tween (PBS-T) and blocked with PBS containing 1.0% BSA. Intestinal washes (100 μl/well) were added to each well and allowed to incubate for 2 h at room temperature. All plates were washed with PBS-T, and bound antibodies were detected with peroxidase-conjugated rabbit anti-chicken IgY or IgA antibodies and 3,3′,5,5′-tetramethylbenzidine substrate (Sigma). Optical density at 450 nm (OD_{450}) was measured with an automated microplate reader (Bio-Rad, Richmond, CA). As negative controls, profilin intestinal antibody levels in non-vaccinated, uninfected chickens were measured. Antibody levels were expressed as ΔOD values (OD_{450} vaccinated, infected group – OD_{450} non-vaccinated, uninfected controls). All samples were analyzed in triplicate.

2.8. Analysis of intestinal intraepithelial lymphocyte (IEL) subpopulations

The duodenum was excised at 3 days post-secondary immunization from 5 chickens per group, cut longitudinally, and washed with ice-cold Hank's balanced salt solution (HBSS) without calcium chloride or magnesium sulfate (Sigma). Single cell suspensions of intestinal IELs were isolated by density gradient centrifugation as described (Lee et al., 2010), resuspended in 1.0 ml of HBSS containing 3.0% fetal bovine serum (FBS) and 0.01% sodium azide and analyzed for expression of chicken

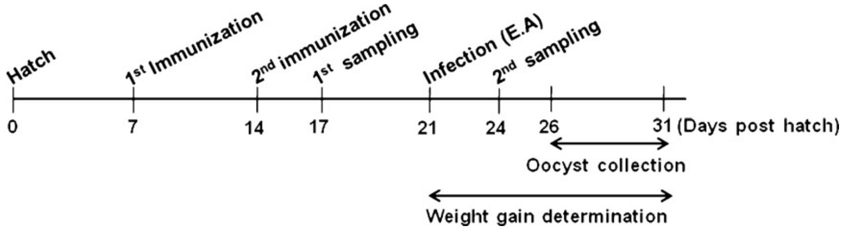

Fig. 1. Schematic outline of the experimental design. Chickens were immunized with profilin plus Montanide adjuvants ISA 71 VG or IMS 1313, or CFA/IFA, at 7 and 14 days post-hatch and infected with *E. acervulina* at 1 wk post-secondary immunization. Intestinal tissues were obtained at 17 and 24 days post-hatch. Body weight was individually assessed between 0 and 10 days post-infection and fecal samples were collected between 5 and 10 days post-infection.

leukocyte surface antigens using a FACSAria II flow cytometer (BD Biosciences, San Jose, CA). Monoclonal antibodies against the following surface markers were used: K55, pan chicken lymphocyte (positive control); K1, chicken macrophages/thrombocytes; CD4, chicken T helper lymphocytes; CD8, chicken cytotoxic T lymphocytes; TCR1, chicken γδ T cell receptor (TCR); TCR2, chicken αβ TCR; BU1, chicken B lymphocytes; and HB2, human T lymphocytes (negative control) (Lillehoj et al., 1988).

2.9. Statistical analysis

All data were subjected to one-way analysis of variance using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL). Mean ± S.D. values of treatment groups were compared using the Duncan's multiple range test and differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Effect of Montanide™ adjuvants IMS 1313 and ISA 71 VG on protective immunity

The measured parameters of disease resistance were augmented weight gain, reduced fecal parasite shedding, and decreased intestinal lesions in coccidia-infected chickens. Birds orally immunized with profilin plus IMS 1313, or subcutaneously immunized with profilin plus ISA 71 VG, exhibited similar increased body weight gains, both being greater when compared with animals nasally or ocularly immunized with profilin plus IMS 1313, or subcutaneously immunized with profilin plus Freund's adjuvant (Fig. 2A). However, the weight gains in the two former groups were lower compared with uninfected controls, suggesting partial protection against the infection. All adjuvant formulations, except for IMS 1313 given by the nasal route, equally decreased fecal oocyst shedding, compared with the unimmunized and infected controls ($p < 0.05$; Fig. 2B). Similarly, all adjuvant formulations, except for IMS 1313 given by the ocular route, equally decreased intestinal lesions, compared with the non-vaccinated, infected controls ($p < 0.05$; Fig. 2C).

3.2. Effect of Montanide™ adjuvants IMS 1313 and ISA 71 VG on profilin-reactive intestinal IgY and sIgA antibody levels

Profilin-specific IgY and sIgA levels were measured in the intestine prior to and following *E. acervulina* infection in chickens given the subunit vaccine in conjunction with various adjuvants. IgY levels were equally increased at 3 days post-secondary immunization (prior to coccidia infection) using the IMS 1313 adjuvant by the nasal and ocular routes, or using ISA 71 VG or Freund' adjuvant given subcutaneously (Fig. 3A). Quite distinctly, IgY levels at 3 days post-infection were increased in birds given oral profilin plus IMS 1313, or ISA 71 VG, compared with the remaining groups ($p < 0.05$; Fig. 3B). For sIgA, CFA/IFA were clearly better than the other adjuvants for increasing the levels of this antibody in the gut prior to parasite infection ($p < 0.05$; Fig. 4A), whereas nasal or ocular IMS 1313 and

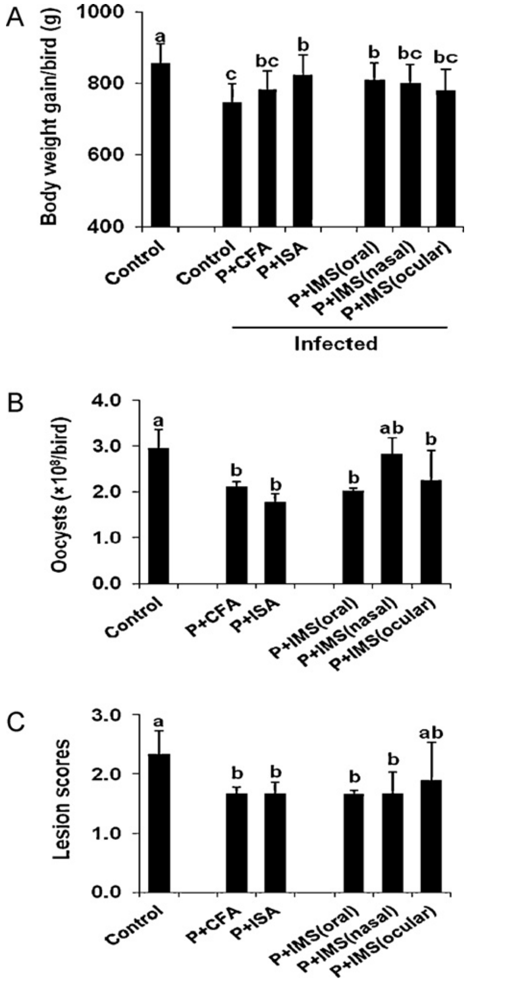

Fig. 2. IMS 1313 and ISA 71 VG enhance protective immunity against experimental avian coccidiosis. Chickens were subcutaneously immunized twice with PBS (control), 50 μg of profilin (P) plus CFA/IFA or ISA 71 VG, or were orally, nasally, or ocularly immunized with 50 μg of profilin plus IMS 1313. At 7 days post-secondary immunization, the animals were left uninfected or infected with 1.0×10^4 *E. acervulina* parasites. Body weight gains (A), fecal parasite excretion (B), and intestinal lesion scores (C) were determined. Each bar represents the mean ± S.D. value ($n = 8$). Within each graph, bars with different letters are significantly different according to the Duncan's multiple range test ($P < 0.05$).

subcutaneous ISA 71 VG were better than oral IMS 1313 or subcutaneous Freund's adjuvant for increasing antibody levels post-infection ($p < 0.05$; Fig. 4B).

3.3. Effect of Montanide™ adjuvants on intestinal IEL subpopulations in uninfected chickens

The expression of an expanded panel of leukocyte surface markers was determined in the gut of uninfected chickens vaccinated with profilin plus adjuvants. As shown in Fig. 5, immunization with profilin in combination with ISA 71 VG was universally better than profilin plus IMS 1313 or CFA/IFA for increasing the percentages of $CD4^+$, $CD8^+$, $BU1^+$, $TCR1^+$, and $TCR2^+$ cells at 3 days post-secondary vaccination ($p < 0.05$). In general, IMS 1313 given

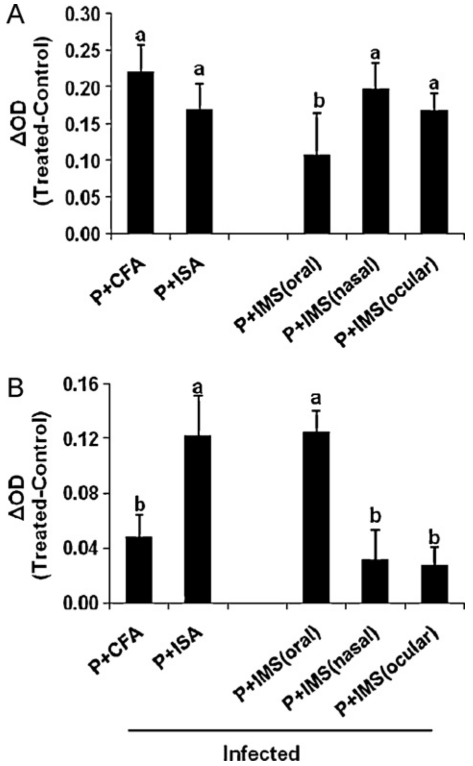

Fig. 3. IMS 1313 and ISA 71 VG increase profilin-reactive intestinal IgY antibody levels during experimental avian coccidiosis. Chickens were subcutaneously immunized twice with 50 μg of profilin (P) plus CFA/IFA or ISA 71 VG, or were orally, nasally, or ocularly immunized with 50 μg of profilin plus IMS 1313. At 7 days post-secondary immunization, the animals were uninfected or infected with 1.0×10^4 *E. acervulina* parasites. Profilin-specific intestinal IgY antibody levels were measured by ELISA at 3 days post-secondary immunization (A) and 3 days post-infection (B). Antibody levels are expressed as ΔOD values (OD_{450} vaccinated and infected group − OD_{450} non-vaccinated, uninfected controls). Each sample was analyzed in triplicate and each bar represents the mean ± S.D. value ($n = 5$). Bars with different letters are significantly different according to the Duncan's multiple range test ($P < 0.05$).

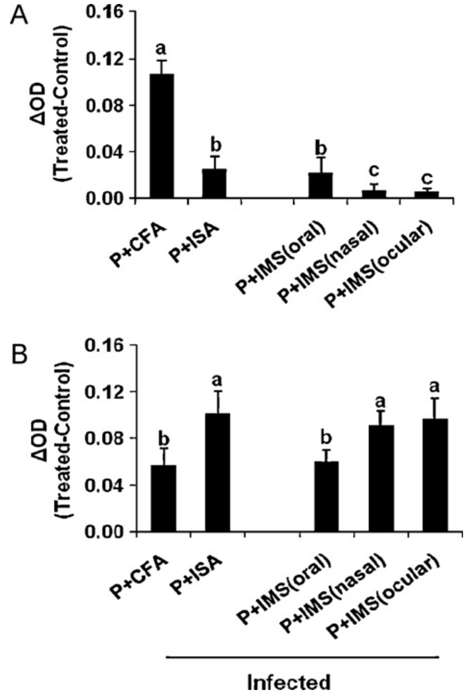

Fig. 4. IMS 1313 and ISA 71 VG increase profilin-reactive intestinal sIgA antibody levels during experimental avian coccidiosis. Chickens were subcutaneously immunized twice with 50 μg of profilin (P) plus CFA/IFA or ISA 71 VG, or were orally, nasally, or ocularly immunized with 50 μg of profilin plus IMS 1313. At 7 days post-secondary immunization, the animals were uninfected or infected with 1.0×10^4 *E. acervulina* parasites. Profilin-specific intestinal sIgA antibody levels were measured by ELISA at 3 days post-secondary immunization (A) and 3 days post-infection (B). Antibody levels are expressed as ΔOD values (OD_{450} vaccinated and infected group − OD_{450} non-vaccinated, uninfected controls). Each sample was analyzed in triplicate and each bar represents the mean ± S.D. value ($n = 5$). Bars with different letters are significantly different according to the Duncan's multiple range test ($P < 0.05$).

by any route of immunization was generally equivalent, or moderately superior to, Freund's adjuvant in increasing the percentages of the denoted cell subpopulations. The only exception was the greater ability of IMS 1313 given ocularly to increase K1$^+$ macrophages/thrombocytes, compared with the other adjuvant groups ($p < 0.05$).

4. Discussion

This study documents the immunoenhancing effects of Montanide adjuvants (aqueously delivered IMS 1313 nanoparticle adjuvant and subcutaneously injected ISA 71 VG water-in-oil adjuvant) on profilin protein vaccination against *E. acervulina* infection. The major findings are: (1) chickens orally vaccinated with profilin plus IMS 1313, or subcutaneously with profilin plus ISA 71 VG, had increased body weight gains compared with animals nasally or ocularly immunized with profilin plus IMS 1313, or subcutaneously immunized with profilin plus Freund's adjuvant, (2) all adjuvants, with the exception of nasally- or ocularly delivered IMS 1313, decreased fecal oocyst shedding and reduced intestinal lesions, compared with the unimmunized, infected controls, (3) chickens immunized with profilin plus IMS 1313 or ISA 71 VG showed higher post-infection intestinal IgY and sIgA responses, compared with the non-vaccinated or profilin/Freund's adjuvant-vaccinated groups, and (4) vaccination with profilin plus ISA 71 VG was more effective than profilin/IMS 1313 or profilin/CFA for increasing the percentages of CD4$^+$, CD8$^+$, BU1$^+$, TCR1$^+$, and TCR2$^+$ intestinal T and B lymphocytes, whereas the K1$^+$ macrophage/thrombocyte subpopulation was increased to the greatest extent following ocularly delivered profilin plus IMS 1313.

Profilin is an actin-binding protein found in all eukaryotic cells that promotes the elongation of actin filaments by delivering monomeric G-actin to the growing filament (Schutt et al., 1993). Apicomplexan protozoa, such as *Eimeria*, lacking profilin retain the ability to grow and replicate, but cannot invade host cells, presumably because their capacity to polymerize actin for host invasion is compromised (Kucera et al., 2010). Therefore, profilin has been considered as a potential vaccine candidate for controlling coccidiosis, malaria, and toxoplasmosis. *Eimeria* profilin was originally identified in the merozoites of the parasite

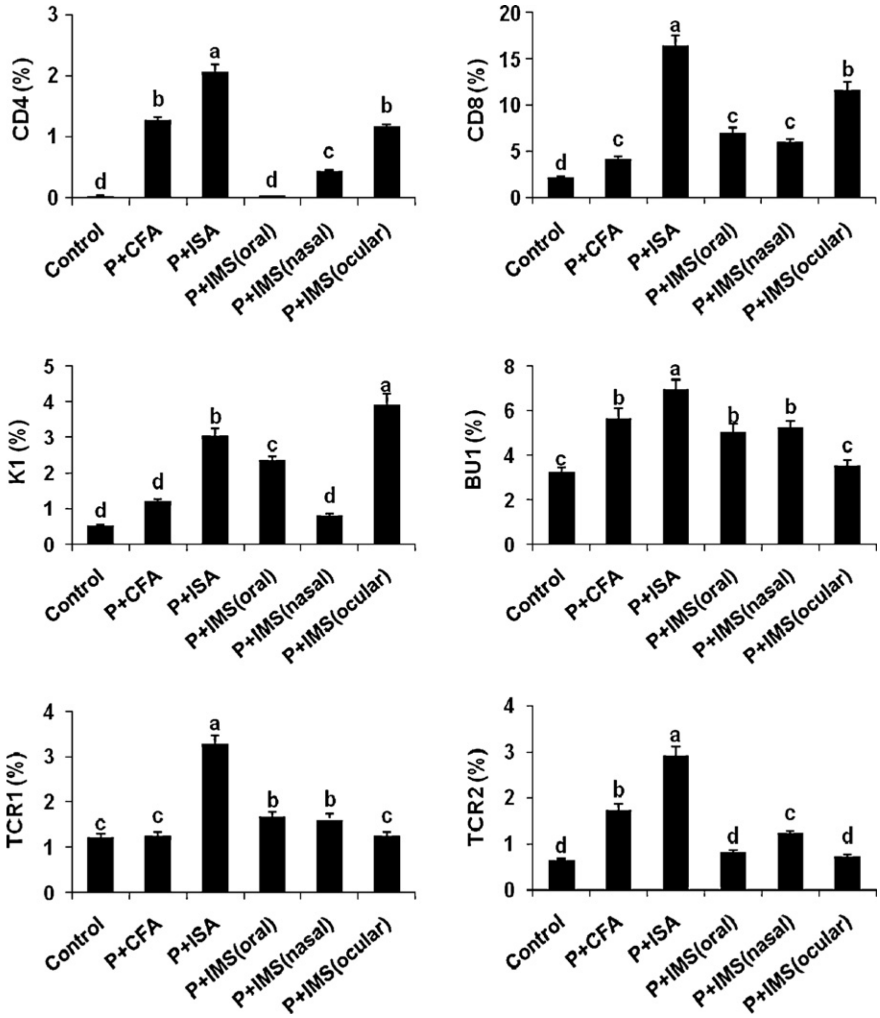

Fig. 5. Effect of IMS 1313 and ISA 71 VG on intestinal IEL subpopulations in uninfected chickens. Chickens were subcutaneously immunized twice with 50 μg of profilin (P) plus CFA/IFA or ISA 71 VG, or were orally, nasally, or ocularly immunized with 50 μg of profilin plus IMS 1313. At 3 days post-secondary immunization, intestinal IELs were isolated and analyzed by a flow cytometery for the percentage of cells expressing the indicated cell surface markers. Each bar represents the mean ± S.D. value ($n = 5$). Within each graph, bars with different letters are significantly different according to the Duncan's multiple range test ($P < 0.05$).

as an immunogenic protein which induced antigen-specific proliferation and interferon (IFN)-γ production by chicken spleen lymphocytes (Lillehoj et al., 2000). Subsequently, *Eimeria* profilin was shown to induce additional inflammatory mediators, including interleukin (IL)-12, monocyte chemoattractant protein-1, IL-6, and tumor necrosis factor-α, and to have potent anticancer and antiviral activities through stimulation of Toll-like receptor 11 (Gowen et al., 2008).

Protective host immunity to avian coccidiosis has been directly correlated with increased post-infection body weight gain, reduced fecal parasite excretion, and decreased incidence and severity of gut lesions (Chapman et al., 2005; Lee et al., 2007; Jang et al., 2010, 2011). In the current study, all of these parameters were altered in a manner consistent with increased disease resistance in animals vaccinated with profilin plus IMS 1313 or ISA 71 VG, compared with unvaccinated controls. Because mucosal immunization with profilin plus IMS 1313 elicited disease protection similar to that produced by subcutaneous vaccination with profilin plus ISA 71 VG, application of the former as an aqueous solution may offer a practical means for large-scale commercial vaccination efforts using drinking water, spray, or eye drop delivery techniques. Future field trial studies with the denoted vaccine/adjuvant/delivery route combinations will be needed to confirm or refute this possibility.

While increased serum IgG antibody levels during natural *Eimeria* infection do not correlate with the level of protection during avian coccidiosis, intestinal IgY and sIgA do play a beneficial role in disease resistance (Lee et al., 2009). During coccidia colonization of the gut, chickens normally produce parasite-specific IgY and sIgA antibodies that reach maximum levels between 7 and 20 days post-infection (Trees et al., 1989; Yun et al., 2000). Chickens vaccinated with profilin plus Freund's adjuvant had increased levels of IgY and sIgA antibodies, compared with vaccination with profilin alone, that were associated with local immune defense against *Eimeria* (Lillehoj et al., 2004; Lee et al., 2009). The results of the present study confirm and extend these earlier observations to now include a mucosal adjuvant, Montanide™ IMS 1313 N VG PR and lend further support for a protective role for parasite-reactive gut antibodies in resistance to chicken coccidiosis.

In addition to locally produced antibodies, the importance of intestinal leukocytes in mediating protection against *Eimeria* infection is well-recognized (Lillehoj and Trout, 1996). $CD4^+$, $CD8^+$, and $TCR1^+$ T cells recruited to the site of *Eimeria* infection are intimately involved in eliciting protective responses against the invading parasites (Lillehoj and Lillehoj, 2000). This protective immunity is due, in part, to the elaboration of proinflammatory cytokines and chemokines that serve to activate local immune cells and recruit additional leukocytes to the gut (Yun et al., 2000; Jang et al., 2010). Moreover, the extent and character of the cell-mediated response depends on the particular species of invading *Eimeria*. For example, an *E. acervulina* infection mainly induces a duodenal $CD8^+$ T cell and macrophage response with increased levels of gut IL-2, IL-4, IL-8, IL-10, and IFN-γ (Cornelissen et al., 2009). Presumably, increased levels of intestinal leukocyte subpopulations following immunization with profilin plus ISA 71 VG, and to a lesser extent in combination with IMS 1313, triggers heightened secretion of cytokines/chemokines that stimulate and amplify gut cell-mediated immunity against the parasite.

The data presented in this report also provide a comparison of the relative efficacy of the Montanide™ adjuvants with Freund's adjuvant, typically regarded as the "gold standard" for adjuvanticity. While highly effective, the use of CFA in humans and veterinary animals is prohibitive due its toxicity. By contrast, the Montanide™ series of immunostimulants are comparatively non-toxic. Based on the findings of our previous investigations (Jang et al., 2010, 2011) and the current studies, it is clear that IMS 1313 and ISA 71 VG are equal to, or in some instances better than Freund's adjuvant for stimulating protective immunity against experimental *E. acervulina* infection and for increasing humoral and cellular immune parameters associated with disease resistance. To the best of our knowledge, this is the first report showing the efficacy of the IMS 1313 adjuvant in commercial meat-type chickens. Since mucosal administration of vaccines as aqueous solutions offers greater advantages over parenteral delivery for immunization against pathogens that invade epithelial surfaces (Vyas and Gupta, 2007), the mode of action of this nanoparticle adjuvant requires additional investigation for potential application by the poultry industry.

In conclusion, this study demonstrates the protective and immune enhancing effects of the Montanide™ IMS 1313 and ISA 71 VG adjuvants for delivery of a recombinant subunit vaccine against avian coccidiosis. Increased post-infection body weight gains, reduced fecal parasite excretion, and decreased gut lesion were observed in chickens vaccinated with profilin plus adjuvants compared with non-vaccinated controls. Increased parasite-specific intestinal antibody levels and augmented intestinal leukocytes subpopulations were generally equal to or greater in animals given profilin plus IMS 1313 or ISA 71 VG compared with profilin plus CFA. Future studies are warranted to elucidate the underlying immune mechanisms modulated by these adjuvants, and to explore their effects on other economically important mucosal pathogens of poultry.

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